Characterization of Conditions Required for X-Ray Diffraction Experiments with Protein Microcrystals

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INTRODUCTION

Synchrotron sources provide a number of well-recognized advantages for protein crystallography experiments. Among the key features one can mention are extremely rapid data collection, routinely higher resolution, the opportunity to tune the x-ray wavelength to an absorption edge for multiple anomalous dispersion (MAD) phasing, and the ability to use much smaller crystals than are practical with a laboratory source.

The ability to conduct diffraction experiments with protein microcrystals can open up a number of important opportunities. Microcrystals can be used to evaluate the quality of diffraction at an early stage, before extensive effort is invested to improve the crystal size. If microcrystals are large enough to allow many diffraction patterns to be collected without severe radiation damage, there is even no need to further optimize the conditions needed to produce larger crystals. These and other reasons for the development of microcrystal diffraction cameras have been discussed recently (1)

Experimental work with protein microcrystals – defined here as crystals that are only a few tens of micrometers on edge – can be facilitated by focusing the x-ray beam to about one tenth the usual size (2,3). Focusing to a smaller size increases the x-ray flux density at the crystal, an important consideration since the exposure required to produce adequate count statistics in the diffraction spots scales inversely with the crystal volume. Use of a narrow x-ray beam, comparable in size to the protein microcrystal itself, also helps to reduce the background scattering from material surrounding the crystal and from the air in the path between the collimator and the beam stop.

Radiation damage ultimately places a limit on the smallest protein crystal size that can be used for high-resolution data collection, however, since a given number of x-rays must pass through a crystal, regardless of its size, in order to produce a desired number of counts in each diffraction spot. Thus, as the number of unit cells in a crystal decreases, the x-ray exposure per unit cell must increase proportionately. Finally, when the crystal size is too small, radiation damage will destroy the crystal before an adequate exposure has been completed (4,5).

In anticipation of work that will be done with protein microcrystals at the Advanced Light Source at the Lawrence Berkeley National Laboratory, we have further characterized the limitation on crystal size that is imposed by radiation damage. We find that crystals of bacteriorhodopsin are severely damaged at resolutions better than 3Å (and significant damage is apparent at lower resolution) after an exposure of about 10^{10} photons/μm^2. The damage "threshold" of 10^{10} photons/μm^2 that we measure at ~3Å resolution is within a factor of two of the value that was estimated from earlier measurements made in electron diffraction experiments (4). As a conservative rule-of-thumb, we estimate that at least one high-resolution diffraction pattern, covering one degree of rotation, can be obtained from a protein crystal if the crystal size (in micrometers) is 1/10 the unit cell size (in Angstroms). Data covering more than 100 degrees of rotation can therefore be obtained if the crystal size (in micrometers) is 1/2 the unit cell size (in Angstroms). The need to minimize the air-scatter background becomes apparent as the crystal size

is reduced to very small dimensions. Replacing the nitrogen cryostream in the region of the sample with a helium cryostream significantly reduces the background.

MATERIALS AND METHODS

Measurements of radiation damage were performed on crystals of the F219L mutant of bacteriorhodopsin, grown by the lipidic cubic phase technique (6). Radiation damage measurements were carried out with equipment set up on the x-ray bend magnet test beam line 7.3.3. This beamline has a toroidal mirror located 16m from the source which collected 3mrad of bending magnet radiation and focused it to a spot on the sample at the 37m location. This mirror provides the full amount of horizontal focusing, resulting in a convergence angle of 2.3 mrads. This mirror provides only a small amount of vertical focusing, however. Most of the vertical focusing is provided by the microfocus mirror located 12 cm before the sample. This mirror was of similar design to those used elsewhere at the ALS (7). The resulting vertical convergence angle onto the sample was 2.5 mrads. A double crystal Ge (111) monochromator, located 1m before the sample, was set to produce 11 keV photons with an energy bandpass ~ 1:1000.

A spot size of 130x54 micrometers (FWHM) with a total flux of 2.2×10^9 photons/s (400mA ring current) was used for the measurements of radiation damage. By means of slits an even smaller spot size of 48x54 micrometers was used to reduce the background scattering for diffraction from very small crystals. A MSC sample cooler was operated at a specimen temperature of ~170K. An ADSC Quantum 4 CCD camera (Area Detector Systems Corp.) was used to record the diffraction patterns. Diffraction patterns were indexed and integrated intensities were produced with the HKL – DENZO software suite (8).

In order to demonstrate the principle for further reducing the air-scatter background, nitrogen gas was temporarily replaced by helium gas in the cold-gas stream of a protein-crystal sample-cooler (Molecular Structure Corporation). We estimate that 2/3 or more of the path length between the tip of the collimator and the beam stop is effectively “purged” by helium gas in this way.

RESULTS

The strategy for our measurement of radiation damage was to collect a 10-degree wedge of data as a series of 10 diffraction patterns, rotating the crystal by 1 degree for each image recorded on the CCD camera. At the end of such a series, the crystal was returned to its original orientation, and the same 10-degree wedge was recollected. This process was continued until considerable deterioration of the diffraction pattern became apparent at high resolution

Figure 1 shows the number of diffraction spots for which I/σ is greater than 5 for three different resolution shells. The number of reflections in each shell is shown as a percentage of the number present in the first 10-degree wedge of data; the absolute number of reflections found in the first wedge of data is shown in parentheses for each resolution shell. This representation of the data is one in which the overall effects of radiation damage are easily appreciated. It seems clear that, in most cases, one would not want to collect data after an exposure of more than 10^{10} photons/μm^2. Thus, this value of exposure is roughly the limit of what can be used to collect data at 3Å resolution. A resolution of 3Å is also about the value obtained in electron diffraction experiments with two-dimensional bacteriorhodopsin crystals. The limiting exposure measured in our experiments with three-dimensional crystals is indeed close to the value of 1.6×10^{10} photons/μm^2 estimated by Henderson (4) to be the maximum acceptable exposure on the basis of electron diffraction data.

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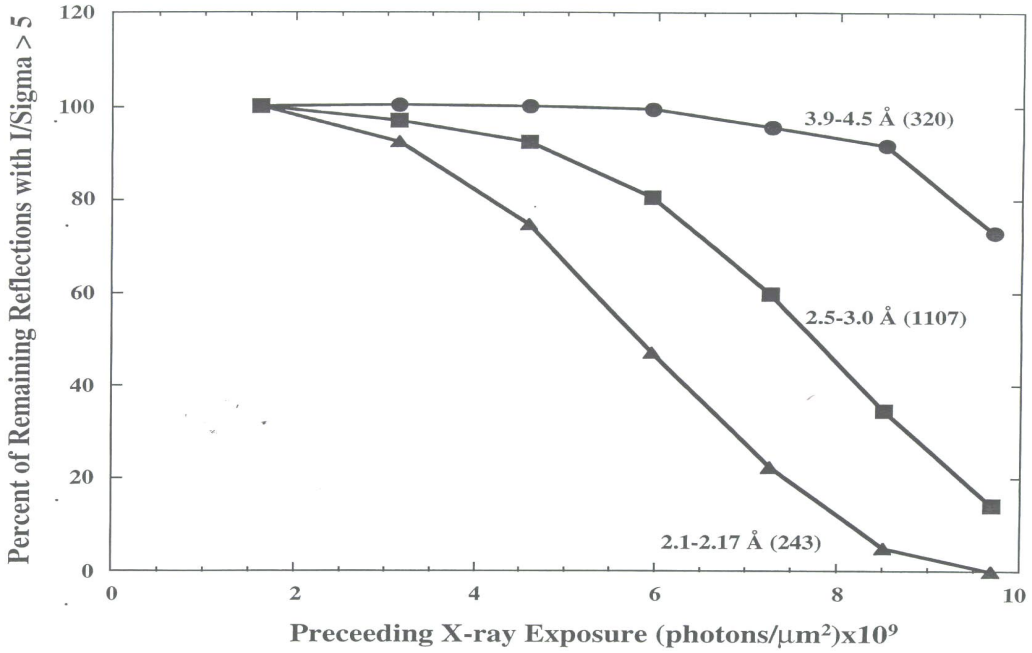

Figure 1. Degradation of crystal quality as a function of x-ray exposure. The number of diffraction spots with $I/\sigma > 5$ within a 10-degree wedge is plotted for three resolution shells, 2.10Å – 2.17 Å, 2.5 Å – 3.0 Å, and 3.9 Å – 4.5 Å. These numbers are obtained from the SCALEPACK intensity statistics by subtracting the number of reflections with $I/\sigma < 5$ from the total number of reflections in a shell. The total number of diffraction spots with $I/\sigma > 5$ that were found within the first 10-degree wedge of data is indicated in parenthesis for each shell, and the number of diffraction spots found for each shell is presented as a percentage of the number found in the first 10-degree wedge. From the slope of the curve at low exposure it is apparent that significant damage is already present at 2Å resolution after an exposure of $2x10^9$ photons/μm^2. Damage is severe at resolution of 3A after an exposure of ~10^{10} photons/μm^2, and it would appear that the crystal would no longer show diffraction at 4 Å resolution after receiving twice that exposure.

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REFERENCES

1. Cusack, S., H. Belrhali, A. Bram, M. Burghammer, A. Perrakis, and C. Riekel. 1998. Small is beautiful: protein micro-crystallography. *Nature Struct. Biol.*, Synchrotron Supplement 634-637.

2. Bilderback, D. H., S. A. Hoffman, and D. J. Thiel. 1994. Nanometer spatial resolution achieved in hard x-ray imaging and Laue diffraction experiments. *Science*. 163:201-203.

3. Pebay-Peyroula, E., G. Rummel, J. P. Rosenbusch, and E. M. Landau. 1997. X-ray structure of bacteriorhodopsin at 2.5 Angstroms from microcrystals grown in lipidic cubic phases. *Science*. 277:1676-1681.

4. Henderson, R. 1990. Cryo-protection of protein crystals against radiation damage in electron and x-ray diffraction. *Proc. R. Soc. Lond.* B 241:6-8.

5. Gonzales, A., and C. Nave. 1994. Radiation damage in protein crystals at low temperature. *Acta Cryst.* D50:874-877.

6. Landau, E. M., and J. P. Rosenbusch. 1996. Lipidic cubic phases: a novel concept for the crystallization of membrane proteins. *Proc. Natl. Acad. Sci.* USA 93:14532-14535.

7. MacDowell, A. A., R. Celestre, C-H. Chang, K. Franck, M. R. Howells, S. Locklin, H. A. Padmore, J. R. Patel, and R. Sandler. 1997. Progress towards sub-micron hard x-ray imaging using elliptically bent mirrors. *SPIE.* 3152:126-135.

8. Otwinowski, Z., and W. Minor. 1997. Processing of x-ray diffraction data collected in oscillation mode. *Methods in Enzymology* (Macromolecular Crystallography; Eds. C.W. Carter and R.M. Sweet) 276 Part A:307-326.

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